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Note

Separation of diastereomers of protected dipeptides by normal-phase high-performance liquid chromatography

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The study of racemization in peptide synthesis has long been of concern to peptide chemists. Usually the extent of racemization was determined by analysing a pair of diastereomeric peptides produced during peptide synthesis by a conventional chemical method. Several methods have been developed for determining the ratio of diastereomers quantitatively, including ^{1}H NMR spectroscopy[1,2] and high-performance liquid chromatography (HPLC)[3,4]. Diastereomeric protected dipeptides can be separated by both reversed- and normal-phase HPLC, whereas diastereomic free peptides are separated by ion-exchange chromatography and determined via the ninhydrin reaction[5,6]. Owing to the greater complication and the possibility of adverse effects on the ratio of diastereomers when one or two deprotecting steps are applied, the direct analysis of protected peptides provides a means of significantly simplifying racemization studies. Systematic studies of the reversed-phase HPLC separation of protected peptides have been reported[3,4,7,8], but there are only a few examples of normal-phase separations[9–12]. In this work we used X–D, L–AA_1–L–AA_2–OBzl as model compounds, where Bzl is benzyl and X could be formyl (For-), acetyl (Ac-), *tert.*-butyloxycarbonyl (Boc-), benzoyl (Bz-) and benzyloxycarbonyl (Z-) groups and AA_1 and AA_2 are Ala and Phe, to study systematically the separation of these diastereomeric pairs by normal-phase HPLC.

EXPERIMENTAL

L-Amino acids were purchased from Kyowa Fermentation (Tokyo, Japan) and D-amino acids from Sigma (U.S.A.). All solvents were obtained from Alps Chemical (Taiwan). Thin-layer chromatography was performed on silica gel GF_{254} (type 60) from E. Merck (F.R.G.). Ninhydrin reagent was used to locate the carboxyl- and N-protected amino acids and synthetic dipeptides were detected by the chlorine–tolidine method[13]. N- or carboxyl-protected amino acids were prepared by conventional methods and protected dipeptides were synthesized by the dicyclohexylcarbodiimidecoupling method.

An HPLC system from Waters Assoc. (Milford, MA, U.S.A.) was used for the analytical separations, consisting of one M6000A solvent-delivery unit and a U6K universal liquid chromatograph injector, coupled to an M450 variable-wavelength UV spectrophotometer and an Omniscribe two-channel chart recorder (Houston In-

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struments, Austin, TX, U.S.A.). Integration was performed electronically with an SP4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.). Diastereomeric dipeptides were separated on a silica gel column (25 cm × 4 cm I.D.) (E. Merck) using 2-propanol (IPA) in *n*-hexane or chloroform as the mobile phase and detected at UV 254 nm.

TABLE I

SEPARATION OF PROTECTED DIASTEREOMERIC PEPTIDES

IPA = 2-Propanol; Hex = *n*-hexane; $CHCl_3$ = chloroform.

Compound		*k'*	*Separation factor (α)*	*Eluent*
Bz–Phe–Phe–OBzl	L–L	2.53	1.58	IPA–Hex(0.9:100)
	D–L	4.00		
Ac–Phe–Phe–OBzl	L–L	3.33	1.40	IPA–Hex(3.6:100)
	D–L	4.66		
For–Phe–Phe–OBzl	L–L	3.66	1.36	IPA–Hex(3.6:100)
	D–L	5.00		
Z–Phe–Phe–OBzl	L–L	4.73	1.27	IPA–Hex(0.6:100)
	D–L	6.00		
Boc–Phe–Phe–OBzl	L–L	4.00	1.25	IPA–Hex(0.5:100)
	D–L	5.00		
Bz–Ala–Phe–OBzl	L–L	4.73	1.23	IPA–Hex(1.5:100)
	D–L	5.80		
Ac–Ala–Phe–OBzl	L–L	3.75	1.15	IPA–$CHCl_3$(3.0:100)
	D–L	3.25		
For–Ala–Phe–OBzl	L–L	2.60	1.21	IPA–$CHCl_3$(4.0:100)
	D–L	2.15		
Z–Ala–Phe–OBzl	L–L	7.13	1.12	IPA–Hex(1.6:100)
	D–L	8.00		
Boc–Ala–Phe–OBzl	L–L	6.00	1.03	IPA–Hex(1.4:100)
	D–L	6.18		
Bz–Phe–Ala–OBzl	L–L	4.27	1.20	IPA–Hex(1.2:100)
	D–L	3.53		
Ac–Phe–Ala–OBzl	L–L	6.33	1.16	IPA–Hex(4.0:100)
	D–L	5.47		
For–Phe–Ala–OBzl	L–L	2.50	1.50	IPA–$CHCl_3$(3.0:100)
	D–L	1.65		
Z–Phe–Ala–OBzl	L–L	5.13	1.26	IPA–Hex(0.7:100)
	D–L	4.06		
Boc–Phe–Ala–OBzl	L–L	7.67	1.31	IPA–Hex(0.6:100)
	D–L	5.87		
Bz–Ala–Ala–OBzl	L–L	4.20	1.50	IPA–Hex(2.0:100)
	D–L	6.33		
Ac–Ala–Ala–OBzl	L–L	2.60	1.13	IPA–$CHCl_3$(7.0:100)
	D–L	2.30		
For–Ala–Ala–OBzl	L–L	3.75	1.19	IPA–$CHCl_3$(7.0:100)
	D–L	3.15		
Z–Ala–Ala–OBzl	L–L	5.00	1.40	IPA–Hex(3.6:100)
	D–L	7.00		
Boc–Ala–Ala–OBzl	L–L	4.80	1.18	IPA–Hex(1.7:100)
	D–L	5.67		

RESULTS AND DISCUSSION

Recently, Benoiton *et al.*[14] used reversed-phase HPLC to separate more than 50 diastereomeric N-protected di-, tri- and tetrapeptide acids and esters and the results were very successful in three quarters of the cases. However, with Phe–Phe and Ala–Phe dipeptides, no separation could be observed and most pairs of diastereomeric peptides were separated with long retention times in the reversed-phase mode, usually more than 30 min and in some instances even longer than 90 min. Therefore, we chose Phe and Ala dipeptides as model peptides for the study of normal phase HPLC.

The data for the separation factors (α) and capacity factors (k') listed in Table I indicate that all pairs of diastereomeric dipeptides except Boc–L, D-Ala–L-Phe–OBzl can be well separated and with short retention times (less than 20 min) by normal phase HPLC (Fig. 1).

This excellent separation method can be used to study racemization in peptide synthesis. Previously reported elution orders of D–L and L–L protected peptides indicated that L–L dipeptides seem to have shorter retention times than D–L dipeptides in reversed-phase HPLC[3,4,7,8]. Our results for the normal-phase HPLC separation of

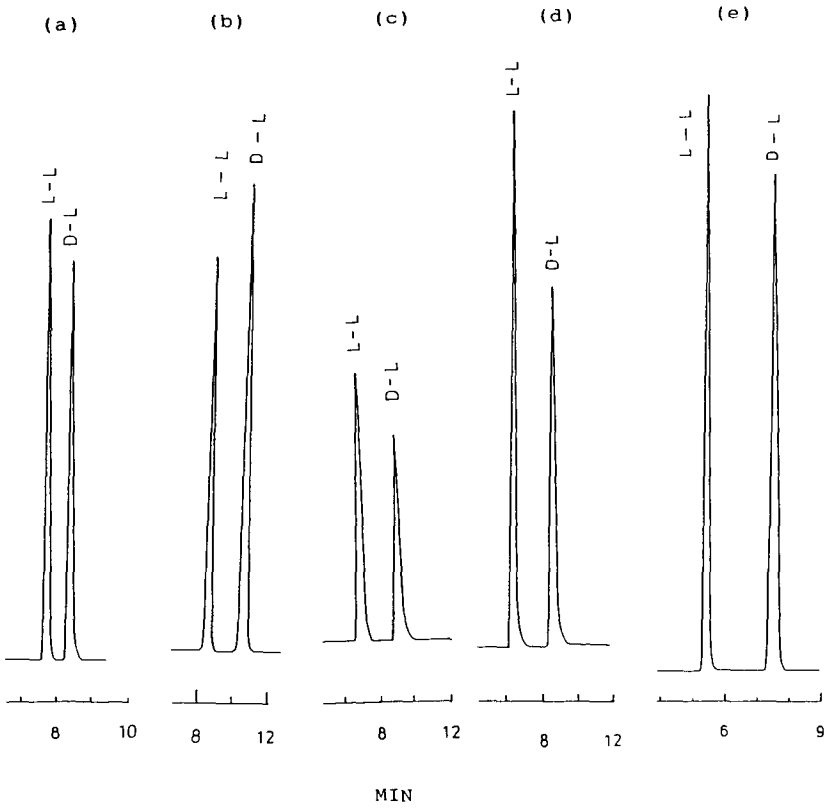

Fig. 1. HPLC profiles of diastereomeric protected dipeptides X–Phe–Phe–OBzl. (a) Boc–D,L-Phe–L-Phe–OBzl; (b) Z–D,L-Phe–L-Phe–OBzl; (c) For–D,L-Phe–L-Phe–OBzl; (d) Ac–D,L-Phe–L-Phe–OBzl; (e) Bz–D,L-Phe–L-Phe–OBzl. Elution conditions as described under Experimental and in Table I.

X–Phe–Phe–OBzl indicated that L–L species always eluted faster than D–L and for X–Phe–Ala–BOzl D–L species have shorter retention times than L–L, regardless of the different protecting groups on the N-terminus or the components of mobile phase. For the other two dipeptides, X–Ala–Ala–OBzl and X–Ala–Phe–OBzl, the N-protecting groups (X) have a critical influence on the elution order: those L–L dipeptides containing bulky N-protecting groups such as Bz, Boc and Z are eluted faster than D–L, whereas L–L dipeptides containing small N-protecting groups such as Ac and For are eluted more slowly than L–L. It is believed that the elution order of diastereomeric protected peptides is influenced by protecting groups, amino acid residues and eluents. This study has demonstrated that in some instances normal-phase HPLC can provide a better means than the reversed-phase mode for separating diastereomeric protected peptides.

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